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Primary cell culture for evaluation of botulinum neurotoxin antagonists☆

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This article is dedicated to the memory of Dr Robert E. Sheridan

Abstract

The actions of botulinum neurotoxin (BoNT) were studied on evoked release of the neurotransmitter glycine in primary mouse spinal cord cells. ^{3}[H]-glycine was taken up by cells in physiological solution and released by depolarization with 56 mM K^{+} in the presence of 2 mM Ca^{2+}. Release of ^{3}[H]-glycine was found to be inhibited by BoNT serotypes A, B and E with similar potency ratios to those observed in the acutely isolated mouse diaphragm muscle. When spinal cord cultures were exposed to BoNT/A for 24 h, inhibition of ^{3}[H]-glycine release was detected at toxin concentrations as low as 10^{-14} M, and complete inhibition was observed at concentration $\geq 10^{-12}$ M. Preincubation of BoNT/A with polyclonal equine antiserum led to antagonism of toxin-induced inhibition of ^{3}[H]-glycine release in spinal cord cells and to protection of mice from the lethal effects of BoNT/A. It is concluded that spinal cord neurons are a useful model for studying botulinum intoxication and for evaluating BoNT antagonists. Published by Elsevier Ltd.

Keywords: Botulinum neurotoxin; Mouse; Antitoxin; Spinal cord; Diaphragm; Muscle tension; Transmitter release

1. Introduction

Botulinum neurotoxins (BoNTs) have emerged in recent years not only as useful medical tools in the treatment of movement disorders but also as potential weapons directed against military forces and civilian populations (Arnon et al., 2001). Universal vaccination could reduce the threat from these agents, but would also limit the therapeutic uses of the toxins. The dichotomy between threat and benefit as well as logistical difficulties with widespread vaccination has raised the need for new therapies for treatment of botulism (Franz et al., 1997; Arnon et al., 2001). The development of pharmacological treatments will require testing in models that reflect the therapeutic potential of candidate drugs in man.

BoNT poisoning inhibits synaptic transmission at skeletal neuromuscular junctions and causes subsequent

Abbreviations: BoNT, botulinum neurotoxin; BSA, bovine serum albumin; HBS, HEPES-buffered saline; i.p., intraperitoneal; PBS, phosphate-buffered saline; SNAP-25, synaptosomal-associated protein of 25 kDA..

☆ Opinions or assertions contained herein are the private views of the authors and are not to be construed as official or as reflecting the views of the Army or the Department of Defense. All research was conducted in compliance with the Animal Welfare Act and other Federal statutes and regulations relating to animals and adheres to principles stated in the Guide for the Care and Use of Laboratory Animals, National Research Council, 1996. The facilities, where this research was conducted are fully accredited by the Association for Assessment and Accreditation of Laboratory Animal Care (AAALAC) International.

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respiratory failure (Sellin, 1985; Simpson, 1986). BoNT toxicity is mediated presynaptically through a series of steps in which cell surface receptors facilitate internalization of circulating toxin with eventual release of an enzymatic light chain into the cytoplasm (Simpson, 2004). The zinc metalloprotease activity of the light chains targets critical proteins required for vesicular neurotransmission: SNAP-25, syntaxin or synaptobrevin (Montecucco and Schiavo, 1993; Schiavo et al., 1995; Foran et al., 1996).

Antitoxin therapies can only be used to neutralize toxin before internalization, but metalloprotease inhibitors offer promise for use at later stages in BoNT intoxication (Sheridan and Deshpande, 1995; Adler et al., 1998, 2000; Schmidt and Stafford, 2002; Anne et al., 2003). In this context, the selection of appropriate model systems is critical for evaluating new therapeutics; survival assays may not be feasible for testing compounds with unknown pharmacokinetics, and cell-free assays cannot adequately address questions of intracellular penetration, biological activity and potential toxicity of therapeutic compounds (Pearce et al., 1997; Adler et al., 1998, 2000; Simpson, 2004; Keller et al., 2004).

Primary cultures of dissociated mammalian spinal cord represent a sensitive system for testing the actions of BoNT and of potential BoNT antagonists on synaptic transmission. Release of radiolabeled glycine or glutamate in response to depolarization is inhibited by tetanus neurotoxin and several BoNT serotypes in a concentration- and time-dependent manner (Williamson et al., 1992, 1996; Keller and Neale, 2001; Keller et al., 2004; Hall et al., 2004). The overall sensitivity of spinal cord cultures to clostridial neurotoxins seems to be greater than that of other cell systems described. Of particular interest is how changes in toxin activity in vitro relate to in vivo survival. The current study compares the efficacy of antiserum in protecting mice from the lethal actions of BoNT/A with its ability to antagonize BoNT/A-mediated inhibition of evoked ^{3}[H]-glycine release in cultured spinal cord cells. The results indicate that inhibition of ^{3}[H]-glycine release is a reliable predictor of survival and suggest that cultured spinal cord cells may be useful for testing potential therapeutic compounds for treatment of BoNT intoxication.

2. Materials and methods

2.1. Spinal cord cultures

Spinal cords were dissected from fetal NIH Swiss mice at gestation day E13, dissociated with trypsin and plated on collagen-coated 12-well culture plates at 10^5 cells/cm^2 (Fitzgerald, 1989). Cells were grown in Eagle's Minimum Essential Medium with 5% heat-inactivated horse serum and a nutrient supplement (N3, Romijn et al., 1981) at 37 °C in 90% air/10% CO_2. Cell cultures were treated with 54 μM 5-fluoro-2′-deoxyuridine and 140 μM uridine from day 5–9 after plating to inhibit glial proliferation. Cultures were fed 1–2 times per week and were used for experiments from 3 to 12 weeks after plating. Cell culture reagents were obtained from Invitrogen Corp. (Carlsbad, CA).

2.2. ^{3}[H]-glycine release

Release of ^{3}[H]-glycine was performed by a method similar to that described by Williamson et al. (1992). The culture medium was removed, and cells were incubated at 37 °C for 30 min in HEPES-buffered saline (HBS) containing 2 μCi/ml ^{3}[H]-glycine. The composition of the HBS was NaCl, 135 mM; KCl, 5 mM; $MgCl_2$, 1 mM; $CaCl_2$, 2mM; glucose, 10 mM; HEPES/NaOH; 10 mM (pH. 7.3) and bovine serum albumin (BSA), 0.1% (w/v). The cells were washed briefly with Ca^{2+}-free HBS and incubated sequentially for 7 min in each of the following modified HBS solutions: 5 mM K^+/0 mM Ca^{2+}, 56 mM K^+/2 mM Ca^{2+} and 5 mM K^+/0 mM Ca^{2+}. High K^+ solutions were osmotically balanced by appropriate removal of NaCl. Each incubation solution was collected, and the radioactivity determined by scintillation counting. Following these incubations, 0.2% sodium dodecyl sulfate was added, and the radiolabel remaining in the cells was determined. Evoked release of neurotransmitter was defined as the quantity of ^{3}[H]-glycine released in the presence of 56 mM K^+/2 mM Ca^{2+} minus the fraction released in 5 mM K^+/0 mM Ca^{2+}. The percent of total release was calculated as evoked ^{3}[H]-glycine release divided by the sum of evoked plus cellular radioactivity.

2.3. BoNT solutions

Solutions of BoNT were prepared in phosphate-buffered saline (PBS) and 0.1% BSA and sterile filtered prior to use. BoNT/A was isolated from Hall strain A as the progenitor toxin with associated hemagglutinin (HA) and non-toxic–nonhemagglutinin (NTNH) proteins (Metabiologics, Madison, WI). The toxin contained 1.3×10^7 mouse i.p. LD50 units/mg protein and had a molecular weight of ~500 kDa. BoNT/B and BoNT/E progenitor toxins were obtained from Wako BioProducts (Richmond, VA) and had molecular weights of 500 kDa and 300 kDa, respectively. BoNT/B and BoNT/E were nicked with trypsin type XI at pH 6 and then treated with an excess of soybean trypsin inhibitor prior to use (Ohishi and Sakaguchi, 1977). Equine antiserum to BoNT/A was an IgG preparation (>95% pure) from PerImmune, Inc. (Rockville, MD), and was raised against the same strain of BoNT/A as that used in these studies. For calculating antitoxin:BoNT/A ratios, the antitoxin was assumed to be a pure IgG of 150 kDa at a concentration of 1 mg/ml in PBS. All standard reagents were obtained from Sigma-Aldrich (St Louis, MO).

2.4. Mouse bioassays

Male CD-1 (ICR) mice (16–22 g upon arrival) were used for in vivo survival and in vitro muscle bioassays of toxin activity as previously described (Sheridan et al., 1999). For muscle contractions, hemidiaphragms with attached phrenic nerves were removed, and the preparations were mounted in a tissue bath at 37 °C in an oxygenated (95% O_2 /5% CO_2) physiological solution consisting of NaCl, 135 mM; KCl, 5 mM; $CaCl_2$, 2 mM; $MgCl_2$, 1 mM; $NaHCO_3$, 15 mM; NaH_2PO_4, 1 mM; glucose 11 mM; pH 7.3. The phrenic nerve was stimulated with brief (0.1 msec) supramaximal pulses, and the resulting muscle twitches were measured with an isometric force transducer and stored on a digital computer for later analysis. For testing of survival, groups of 10 mice were injected i.p. with a 30 LD50 dose of BoNT/A to which antisera were added to generate antibody:BoNT/A ratios ranging from 1:1 to 1000:1. BoNT/A was incubated with antibody for 30 min, and a fixed volume of 0.2 ml was injected per mouse. Animals were followed for 5 days postinjection to allow for a clear assessment of long-term survival with antiserum.

3. Results

3.1. Ca^{2+}- and depolarization-dependent ^{3}[H]-glycine release

Incubation of cultured mouse spinal cord cultures with BoNT/A, BoNT/B or BoNT/E for 24 h at 37 °C profoundly depressed the Ca^{2+}- and depolarization-dependent release of ^{3}[H]-glycine. Each of these toxin serotypes inhibited evoked ^{3}[H]-glycine release almost completely when incubated at sufficiently high concentrations for 6–24 h. Selection of an intermediate concentration of each toxin serotype, however, produced a graded inhibition of release.

As indicated in Fig. 1, the concentration of each serotype required to produce approximately half maximal inhibition of the evoked ^{3}[H]-glycine release was 0.5 pM for BoNT/A, 10 pM for BoNT/B and 2 pM for BoNT/E. When tested on isolated mouse hemidiaphragm muscles, the same rank order of potency was noted, as indicated in the inset to Fig. 1. The concentrations of toxin required to produce 50% muscle paralysis in 1 hr was 25 pM for BoNT/A, 200 pM for BoNT/B and 40 pM for BoNT/E.

3.2. Inhibition of evoked ^{3}[H]-glycine release by BoNT/A

Inhibition of evoked neurotransmitter release by BoNT/A is illustrated in Fig. 2. Incubation of spinal cord cultures for 24 h with BoNT/A resulted in inhibition of ^{3}[H]-glycine release that was well described by a sigmoid curve with a Hill coefficient of 1 and an IC50 of 75 fM. Sensitivity to BoNT/A increased with time in culture, and a three-fold difference in IC50 values was observed between 3- and 12-week cultures. To maximize reproducibility in assays, comparisons of toxin sensitivities were restricted to cultures from a single preparation, which were assayed at the same time after plating.

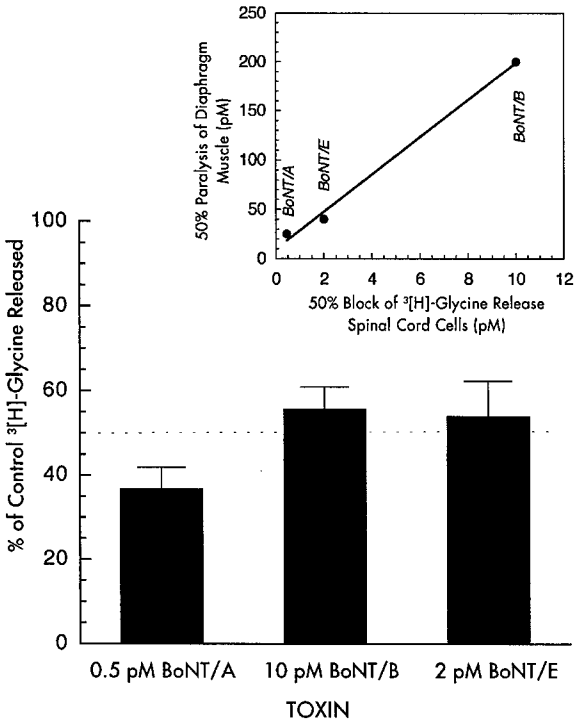

Fig. 1. BoNT concentrations required for ~50% block of evoked neurotransmitter release in primary mouse spinal cord cultures. Replicate cultures were exposed to BoNT for 24 h and assayed for depolarization- and Ca^{2+}-dependent release of ^{3}[H]-glycine. The ordinate represents evoked ^{3}[H]-glycine released in the presence of BoNT relative to the fraction released in the absence of toxin. The symbols are mean ± SE of data from 3 to 6 experiments. Each serotype produced a significant inhibition of ^{3}[H]-glycine release ($P < 0.01$, Students t-test, Graphpad Prism). The inset shows the relation between the BoNT concentration required to inhibit indirect muscle contraction by 50% and that required for 50% inhibition of evoked ^{3}[H]-glycine release in the spinal cord assay. Muscle tensions were assessed at 1 h and ^{3}[H]-glycine release was examined at 24 h after BoNT exposure.

The duration of BoNT incubation with the spinal cord cells was also found to affect the apparent IC50 value. For cultures of the same age, longer incubation with a given BoNT concentration resulted in more profound inhibition of evoked ^{3}[H]-glycine release (Fig. 3, circles). Matched cultures of spinal cord neurons were exposed to BoNT/A, and evoked ^{3}[H]-glycine release was examined at 6, 24, 48 and 72 h after application of the toxin. For each incubation time, a concentration vs. evoked transmitter release curve similar to Fig. 2 was constructed, and the IC50 values were determined. As indicated in Fig. 3, the concentration of toxin required to produce a 50% block of evoked release decreased exponentially between 6 and 48 h, exhibiting an

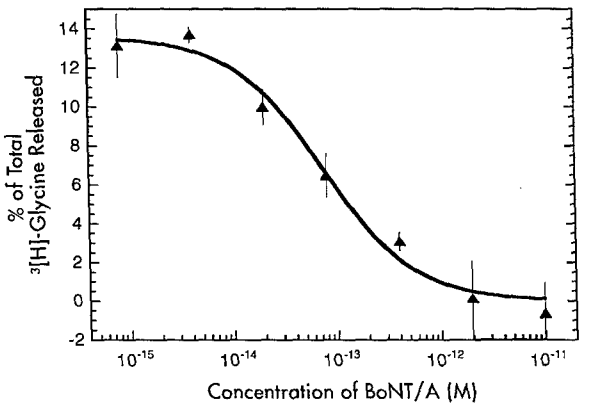

Fig. 2. Inhibition of evoked ^{3}[H]-glycine release in cultured mouse spinal cord neurons as a function of BoNT/A concentration. Evoked release of ^{3}[H]-glycine was measured after a 24-h incubation in culture media at the indicated BoNT/A concentrations. Symbols represents the percent of total ^{3}[H]-glycine released (mean ± SE) from 3 culture dishes at each concentration of toxin. The data were fit with a sigmoid curve with a Hill coefficient of 1 and an IC50 of 75 ± 16 fM. Cultures used in this experiment were grown for 12 weeks prior to assay.

increase in sensitivity to toxin of nearly three orders of magnitude. However, between 48 and 72 h, little or no time-dependence in IC50 values was observed.

3.3. Stability of BoNT in culture media

To eliminate the possibility that the lack of change in BoNT/A sensitivity between 48 and 72 h represented degradation of the applied toxin, the biological activity of BoNT/A was monitored under standard cell culture conditions. Aliquots of 375 pM BoNT/A were incubated in complete media at 37 °C. At various times between 6 and 72 h, samples of toxin were removed, diluted to 50 pM and applied to isolated mouse phrenic nerve-hemidiaphragm preparations. The time required to produce 50% paralysis in the muscles was determined and plotted (Fig. 3, squares). Loss of biological activity in the toxin would be indicated by an increase in the time required to paralyze isolated muscle preparations. As is clear from Fig. 3, there was no loss of BoNT/A activity during 72 h in cell culture medium. Therefore the decrease in the time-dependence of the IC50 between 48 and 72 h must represent attainment of steady state conditions of BoNT action on neurotransmitter release mechanisms and cannot be attributed to a reduction in toxin potency.

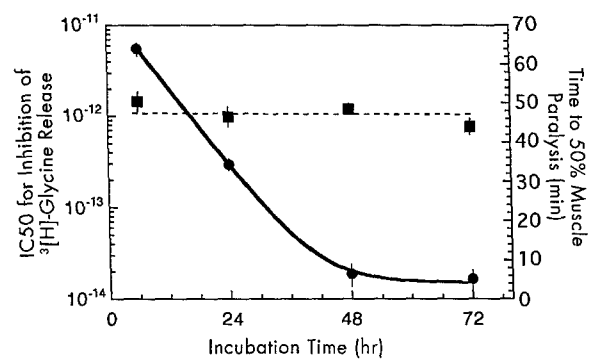

Fig. 3. Dependence of IC50 on BoNT/A incubation time. Evoked release of ^{3}[H]-glycine was measured after cultures were exposed to BoNT/A for the indicated times (●). The data represent the mean ± SE from 3–6 experiments. At the end of incubations, media from spinal cord cultures were removed and assayed for BoNT activity by monitoring the time required to reach 50% muscle paralysis in isolated phrenic nerve-hemidiaphragm preparations (■, mean ± SE, $n=3$). BoNT/A was stable for at least 72 h with no detectable loss in activity under these culture conditions. Reductions in toxin activity would be indicated by increases in the time to reach 50% muscle paralysis.

3.4. Relationship between BoNT/A actions on spinal cord cells and animal survival

Comparisons between the sensitivity of the spinal cord cultures to BoNT/A and survival of mice injected with BoNT/A were made with the same lot of toxin. BoNT/A had a toxicity of 1.3×10^{7} mouse LD50 units per mg protein in the in vivo assay. The same toxin also exhibited an IC50 at 48–72 h of 19 ± 5 fM in cultured spinal cord preparations (Fig. 3). With a molecular weight of 500 kDa for BoNT/A progenitor toxin and a volume of distribution in mice equal to extracellular water (0.2 l/kg), one LD50 of the toxin should correspond to a BoNT/A concentration of 38 fM in a 20-g mouse. Thus the limiting sensitivity of the spinal cord culture with a 48–72 h incubation time (similar to that used in the mouse survival assay) is equal to ~0.5 mouse LD50 units.

3.5. Effect of BoNT/A antiserum

There are currently no drugs for the treatment of BoNT intoxication. Consequently, an antitoxin of known therapeutic potential against BoNT/A in vivo was used to evaluate the predictive value of the mouse spinal cord culture as a model for the efficacy of BoNT therapeutics in animal survival studies. Ideally, the degree of protection afforded by the antitoxin and the relative concentration of antitoxin required in the release assay would closely approximate that required for survival of mice in vivo. To compare the efficacy of the antitoxin used, molar ratios of antibody:BoNT/A ranging from 1:1 to 1000:1 were injected into mice at a dose of toxin equivalent to 30 LD50 units. The resulting survival observed, indicating effective neutralization of BoNT/A, is shown in Fig. 4 (triangles). Doses of antitoxin of approximately 1000 times the toxin dose were required for full protection of the animals.

Similar experiments on spinal cord cultures used a BoNT/A challenge of 1 pM for 24 h which was equivalent

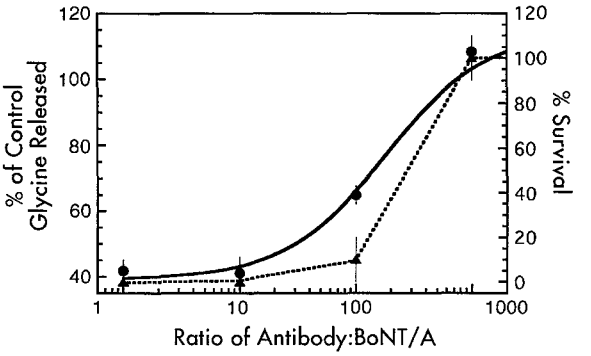

Fig. 4. Effect of BoNT/A polyvalent equine antitoxin IgG on mouse survival (▲) and inhibition of evoked ^{3}[H]-glycine release in cultured spinal cord neurons (●). In each case, BoNT/A was preincubated with antitoxin for 30 min prior to use. In vivo data are expressed as the percent survival in mice injected with a 30 LD50 dose of BoNT/A with the indicated molar ratios of equine antitoxin. Ten mice were used for each data point. The spinal cord response shows release of ^{3}[H]-glycine in the presence of 1 pM BoNT/A for 24 h at the indicated antibody:BoNT/A molar ratios relative to release from control cultures. Triplicate cultures of were used for determinations of ^{3}[H]-glycine release.

to the 30 LD50 challenge dose given to the mice. This toxin challenge resulted in a 60% inhibition of evoked neurotransmitter release. Antitoxin concentrations of 1000 times the toxin concentration were required for full neutralization and preservation of normal levels of evoked ^{3}[H]-glycine release in the presence of BoNT/A (Fig. 4, circles). These results agree closely with those of Hall et al. (2004).

4. Discussion

The long-term culture of primary mouse spinal cord cells has been used for a number of years to study synapse formation and function (Ransom et al., 1977; Bigalke et al., 1985; Williamson et al., 1992, 1996). Evoked release from cultured spinal cord cells can be measured quantitatively using elevated K^{+} to release radiolabeled neurotransmitter. It has been established that under the conditions used in the present studies, release of ^{3}[H]-glycine evoked by depolarization in high K^{+} is specific to neurons, and that the substance released is authentic glycine and not a metabolite (Williamson et al., 1992). As indicated in Fig. 1, evoked transmitter release can be blocked by exposure to several serotypes of BoNT. Further, the rank order potency of the spinal cord cultures to the BoNT serotypes closely resembles the sensitivity of the mouse diaphragm muscle to the same toxins.

The inhibition by BoNT of evoked neurotransmitter release in the spinal cord cultures is a graded function of the toxin concentration that is essentially complete in the presence of 2 pM BoNT/A after 24 h of incubation (Fig. 2). The overall sensitivity of the cultured spinal cord neurons to BoNT is unusually high relative to that of other cell systems studied; significant inhibition of evoked release in the former was evident even at 20 fM BoNT/A (Fig. 2). The conditions necessary for exploiting this sensitivity require that the age of cultures and especially the duration of toxin exposure be controlled to ensure consistency (Fig. 3). The greater sensitivity of spinal cord cultures to BoNT compared to other cell culture systems is due presumably to a higher density of productive toxin receptors on the cell surface (Keller et al., 2004). Their higher sensitivity relative to that of the isolated hemidiaphragm (Fig. 1, inset) is likely due to the longer incubation of spinal cord cells to BoNT, leading to continued internalization of active toxin (Sheridan et al., 1999).

Even more important than the absolute sensitivity of the spinal cord cultures to BoNT is their potential utility for testing therapeutic agents. Currently, the only therapeutic candidates with demonstrated efficacy in vivo are serotype-specific antitoxins. As indicated in Fig. 4, incubation with a BoNT/A specific IgG was able to completely prevent toxin-induced block of neurotransmitter release. The relative concentrations of antitoxin required to effectively neutralize BoNT/A were equivalent to those needed to protect against the lethal effects of the same toxin in vivo. The value of bioassays for BoNT depends on their strengths and weaknesses in the context of the overall use for which the assay is being employed. Based on the current study, primary spinal cord cultures will be invaluable for testing and evaluating future therapeutic candidates for treatment of BoNT intoxication.

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